01/04/2024

Open Isothermal Cloning™ and Platinum Gate Cloning™ T5-SfiI™: Formulation, Usage Protocol and Donor DNA Design.

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Introduction:

Open Isothermal Cloning™ is a long homology DNA assembly method for constructing plasmids using PCR products amplified to include 20-40 bp of overlap to the neighboring DNA molecule. The method eliminates the need for restriction enzymes and produces a seamless DNA construct that consists of the desired sequence of interest without cloning scars like ATT sites or endonuclease binding sites. The reaction is conducted as a one-pot reaction where the donor DNA and a linear destination plasmid are added to an enzyme cocktail that mediates the recombineering of a new molecule. One or multiple fragments can be cloned into a vector to build constructs >100 kb. Open Isothermal Cloning™ uses a high-fidelity archaeal polymerase with 3'-5' exonuclease activity and this confers the reagent terminal mismatch correction, allowing for DNA fragments excised from a plasmid to be used since the polymerase removes up to 10 nt as measured on the 3' strand.

Platinum Gate™ Cloning T5-SfiI™ is a DNA assembly system for recombineering multiple plasmids into virtually any destination vector. Destination vector selection is only limited to sequences with 2 or more SfiI sites. The system is a Golden Gate-like DNA assembly system that assembles multiple donor fragments excised from a plasmid into a linear destination plasmid by long homology hybridization between donor fragments and the terminal ends of the destination plasmid. The advantage of this system over Golden gate is that domestication of a DNA sequence of interest is a rare even compared to the domestication frequency required for PaqCI (1/(4^7)), SapI (1/(4^6)), and BsaI and BsmBI 1/(4^6). The domestication of 1 in every 4^8 reduces the need to modify a sequence for recombineering purposes. The system is simpler in cloning design with the same power as Gateway assembly since it only relies on long homology for cloning instead of ATT sites.

The enzymatic cocktail consists of a high-temperature DNA ligase like Taq or Tth ligase, a high-fidelity polymerase, and T5 exonuclease. The cloning process starts when T5 exonuclease chews back the 5' strand creating 3' overhangs up to 80 nt long. 20-40 nt with homology with a neighboring fragment (also called sticky end) on the 3' overhang mediate hybridization between two cognate DNA fragments by Watson and Crick-based hybridization. The high-fidelity polymerase first removes the first 10 nt on the 3' strands of hybridizing fragments and extends the strand into the 5' nt of the cognate DNA. The high-temperature DNA ligase then covalently associates the linear DNA into a circularized vector. Inclusion of SfiI into this reagent is possible because SfiI has 100% activity in PCR buffer.

Preparing the 5x Isothermal Reaction Master Mix

- 1 M Tris-Hcl (pH 7.5) - 2 ml
- 1 M MgCl2 - 200 µL
- 100 mM dGTP - 40 µL
- 100 mM dATP - 40 µL
- 100 mM dTTP - 40 µL
- 100 mM dCTP - 40 µL
- 1 M DTT - 200 µL
- PEG-8000 - 1.0 g
- 100 mM NAD - 200 µL
- ddH2O - to 4 ml Final Volume

1) Mix dNTPs, NAD+, Tris-HCl, MgCl2 and DTT.
2) Slowly add PEG-8000 to the mixture and mix well, until completely dissolved.
3) Add H2O to a final volume of 4 mL.
4) Prepare aliquots of the 5x isothermal buffer as required.
5) Store at -20 ˚C.

1.33x Assembly Master Mix (25 x 15 µL aliquots):

- 5X Isothermal Master Mix - 100 µL
- Taq DNA Ligase (40 U/µL) - 50 µL
- T5 Exonuclease (1 U/µL) - 2 µL
- Q5 DNA Pol (2 U/µL) - 6.25 µL
- **FOR PLATINUM GATE CLONING T5-SfiI:** SfiI (20 U/µL) - 5-10 µL
- H2O - 216.75 µL (for OIC) or **206.75 (T5-SfiI) @0.4 U/µL or 211.75 (T5-SfiI) @0.2 U/µL**
- Total Volume - 375 µL

1) Work on ice. Mix H2O and 5x buffer, then add enzymes.
2) Prepare 25 x 15 µL aliquots in PCR tubes. Store at -20 ˚C.

These aliquots are concentrated at 1.33 x - add your DNA in a volume of 5 µL to a final volume/concentration of 20 µL / 1x.

1x isothermal reaction buffer:

- 25% PEG-8000
- 500 mM Tris-HCl pH 7.5
- 50 mM MgCl2,
- 50mM DTT
- 5mM NAD
- 1mM each of the four dNTPs

- *Alternative reaction buffer: 1X Isothermal Buffer #1: 20 mM Tris-HCl, 10 mM (NH4)2SO4 10 mM KCl, 2 mM MgSO4, 0.1% Triton® X-100 (pH 8.8 @ 25°C), 5mM NAD, 50mM DTT, 1mM each of the four dNTPs.*

1x Master Mix:

- 5x isothermal buffer: 1X
- Taq ligase: 4 U/µL
- T5 exonuclease: 4 U/mL
- Q5 Pol: 25 U/mL
- **FOR PLATINUM GATE CLONING T5-SfiI: 0.2- 0.4 U/µL of SfiI.**

`Store reaction buffers and master mix at -20 C.`

Isothermal Assembly Protocol.

1. Generate your linear donor DNA and destination plasmid by PCR. Purify reaction by PCR or gel purification kit.
2. Thaw a 15 µl assembly mixture aliquot and keep on ice until ready to be used.
3. Add 5 µl of DNA to be assembled into the master mixture.
    - The DNA should be in equimolar amounts. Use 50-100 ng of linear destination vector DNA fragment.
    - Inserts should be at a 1:1 molar donor-to-destination plasmid ratio.
    - **For T5-SfiI add 10-50 ng of destination plasmid with a 3:1 donor plasmid to destination plasmid ratio.**
4. Incubate at 50 °C for 15 to 60 min (60 min is optimal).
5. Transform into chemically Competent Cells.
6.

Donor DNA Design and Generation by PCR for Open Isothermal Cloning

This protocol outlines the steps for designing and generating donor DNA fragments specifically optimized for Open Isothermal Cloning (OIC). OIC utilizes a single-step isothermal reaction at 50°C, offering greater convenience and potentially higher fidelity compared to traditional PCR-based cloning methods.

1. Design your donor DNA for OIC:

- Identify the desired sequence: Decide on the DNA sequence you want to introduce into your target vector. It can be a gene, regulatory element, or any functional segment.
- Flanking sequences: Include both 5' and 3' flanking sequences homologous to the insertion site in your target vector. These should be longer than standard restriction enzyme overhangs (~20-40 bp) to enhance OIC efficiency.
- Primer design: Design complementary primers that flank your desired sequence and add the homologous regions. Online tools like Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) or NEB Tm Calculator (https://tmcalculator.neb.com/) can assist in primer design.
- **For T5-SfiI special donor plasmid backbone is required and should be added to the donor DNA by TA cloning into the specialized . It is advised that the initial PCR for generating**

the cloning material should be performed with a high fidelity polymerase like Deep Vent or Q5 polymerase. After the PCR reaction, the product should be silica column purified. The PCR product should be incubated with Taq or Tth polymerase to amend an a-tail for TA cloning into a T5-SfiI. The T5-SfiI plasmid backbone is unique because it contains 4 SfiI restriction sites that are optimized for maximum SfiI cleavage.

2. Optimize your PCR reaction:

- Template DNA: Choose a suitable template DNA source containing your desired sequence as in the standard PCR protocol. Genomic DNA, cDNA, plasmid DNA, or synthetic oligonucleotides are all viable options.
- Amplify your donor DNA for OIC using the protocol for your polymerase reagent.

4. Purify your PCR product:

- Choose a suitable purification method based on your downstream application. Similar to standard PCR, options include:
  - Agarose gel electrophoresis and gel extraction
  - PCR clean up kit
  - Magnetic bead-based purification

5. Verify your donor DNA:

- Analyze your purified PCR product by agarose gel electrophoresis to confirm the size and purity of the fragment.
- Consider sequencing your PCR product to ensure sequence fidelity and the absence of unwanted mutations.
- UV Spec or Qubit Fluorometric Quantification for DNA concentration.

pFA.TA Cloning.SfiI Entry-RNA-Sanger: The entry plasmid for Platinum Gate™ Cloning T5-SfiI™.

To produce linear DNA with 3’ T overhangs, digest with HphI.

> **pFA.TA Cloning.SfiI Entry-RNA-Sanger**

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ggccGATTAggccGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
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